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Antimicrobial decapeptide KSL-W attenuates *Candida albicans* virulence by modulating its effects on Toll-like receptor, human β-defensin, and cytokine expression by engineered human oral mucosa

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ABSTRACT

We investigated the toxicity of synthetic antimicrobial decapeptide KSL-W on normal human gingival epithelial cell cultures, its effect on *Candida albicans* adhesion and growth, and the activation of epithelial cell innate immunity. Our results indicate that KSL-W had no toxic effect on cell adhesion or growth, suggesting its safe use with human cells. Pre-treating *C. albicans* with KSL-W attenuated the yeast's virulence as demonstrated by its reduced adhesion and growth on engineered human oral mucosa epithelium and the subsequent decreased expression of some innate defense molecules by targeted epithelial cells. Indeed, the expression of Toll-like receptors and human β-defensins was reduced in tissues infected with KSL-W-treated *Candida*. Proinflammtory cytokine secretion (IL-1β and IL-6) by the epithelial cells was also regulated by KSL-W in a manner similar to that of antifungal molecule amphotericin B. These findings therefore show that KSL-W is safe for use with human cells and is able to attenuate *Candida* virulence by modulating its effects on host innate immunity. This study proposes the potential application of KSL-W peptide as an alternative antifungal agent.

1. Introduction

One of the most commonly encountered opportunistic microorganisms in humans is *Candida albicans* [35], a yeast that is part of the normal microbial flora found on mucosal surfaces such as those of the oral cavity, gastrointestinal tract, and vagina in human beings and domestic animals. This ubiquitous fungus is the most common cause of mucosal and invasive fungal infections observed in humans [29]. Host protection against *Candida* infection is complex and includes different subsets of the immune defense system [5,36,38,40].

Several recent studies have highlighted the significant role played by the innate immunity of epithelial cells in preventing *Candida* infection of the oral mucosa. Primary epithelial cells obtained from various sources [8,38,40] inhibit *C. albicans* growth through cytokine and antimicrobial peptide expression [17,30,38]. The detection of and response to microbial infection by epithelial cells is largely dependent on a family of pattern-recognition receptors called Toll-like receptors (TLRs) [41] which play an active role in recognizing pathogens of considerable target specificity [26]. This recognition produces a series of signaling events that result in the onset of acute host responses to ultimately contribute to the killing of the pathogens [1]. Also demonstrated was the crucial involvement of TLRs in the recognition of fungal pathogens such as *C. albicans*, although less is known regarding the function of these receptors following *Candida* infection [18,25]. The *Candida*-killing activity of epithelial cells involves recognition of the organism by TLR2 and TLR4 and the subsequent activation of NF-κB [32].

Cell-mediated immunity (CMI) against *Candida* infection involves various T lymphocyte subsets [15,36] that may dictate the outcome of the infection. For example, Th1-type responses are associated with resistance to gastrointestinal and systemic *Candida* infections in animals, whereas Th2-type responses are associated with susceptibility to the yeast infection [33]. CMI response against *Candida* infection can be enhanced by humoral defense activity mediated by *Candida*-specific antibodies, such as IgM, IgG, and IgA [5].

A decrease in host defenses may lead to increased susceptibility to *Candida* infections [35,40] which constitute a significant public health challenge of high medical and socioeconomic importance. Although *Candida* infections are treatable with various available drugs, such as polyenes (amphotericin B or nystatin), azoles (voriconazole and posaconazole), and echinocandins (caspo-

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fungin, micafungin, anidulafungin) [21], the effectiveness of several of these antifungal drugs is hampered by undesirable side effects and the emergence of microbial resistance [7,13]. Antifungal drug resistance has thus become a major concern for a growing number of immunocompromized individuals which has resulted in a dramatic increase in the incidence of opportunistic and systemic fungal infections. These facts have generated greater interest toward developing new antifungal drugs using various synthetic and naturally occurring antimicrobial molecules.

Natural antimicrobial peptides, such as defensins produced by epithelial cells, are capable of a broad spectrum of antimicrobial characteristics and may play a role in preventing microbial infections [8,19,45]. Generally, these antimicrobial peptides exhibit selective toxicity for microorganisms and are less likely to induce microbial resistance. In the pursuit of an alternative antifungal treatment, we developed a synthetic α-helical antimicrobial decapeptide, KSL (KKVVFKVKFK), and its analog KSL-W (KKVVFWVKFK) [31], that not only display a wide range of antibacterial activity but were shown to effectively kill selected strains of non-oral and oral pathogens, including *streptococci mutans*. In combination with a surface-active agent, benzalkonium chloride, the peptide significantly reduced *in vitro* biofilm growth [9,10,23,24].

We therefore sought to determine the potential of our synthetic antimicrobial peptides, with a focus on the use of KSL-W (a structurally more stable analog of KSL [31]) to control *Candida* pathogenesis using engineered human oral mucosa as the target host tissue. We also examined the toxicity of the peptide on oral mucosa using gingival epithelial cell cultures.

2. Materials and methods

2.1. Human gingival epithelial cell and fibroblast cultures

Normal human gingival cells (epithelial cells and fibroblasts) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DME, Invitrogen Life Technologies, Burlington, ON, Canada) supplemented with fetal bovine serum to a final concentration of 10% (FBS, Gibco, Burlington, ON, Canada), penicillin and streptomycin (20 μg/ml), and fungizone (0.5 μg/ml). The epithelial cells were cultured in Dulbecco's modified Eagle's (DME) – Ham's F12 (3:1) (DMEH) with 5 μg/ml of human transferrin, 2 nM 3,3′,5′ of triiodo-L-thyronine, 0.4 μg/ml of hydrocortisone, 10 ng/ml of epidermal growth factor, penicillin and streptomycin, and 10% FBS (final concentration). The medium was changed three times a week. When the cultures reached 90% confluence, the cells were detached from the flasks using a 0.05% trypsin-0.1% ethylenediaminetetraacetic acid (EDTA) solution, washed twice, and resuspended in DMEM (for the fibroblasts) or DMEH-supplemented medium (for the epithelial cells). These were then used for subsequent experiments.

2.2. Engineered human oral mucosa (EHOM) tissue

The EHOM model was produced as previously described [8,37]. Briefly, gingival fibroblasts and epithelial cells were used to form a complex three-dimensional spatial cellular organization similar to that found in normal human oral mucosa. The lamina propria was produced by mixing Type I collagen (Gibco-Invitrogen, Burlington, ON, Canada) with gingival fibroblasts, followed by culture in 10% FBS-supplemented medium for four days. The lamina propria was then seeded with gingival epithelial cells to obtain the EHOM. The tissue specimens were grown under submerged conditions until the total surface of the lamina propria was covered with epithelial cells. To produce stratified epithelium, the EHOM was raised to an air–liquid interface for four more days to facilitate the organization of the epithelium into its different strata.

2.3. Effect of KSL-W on gingival epithelial cell adhesion

Epithelial cells (10^4 cells/well) were plated onto tissue culture-grade cover glasses in six-well plates. Immediately after seeding, various concentrations (0, 12.5, 50, and 100 μg/ml) of KSL-W were added to the medium and cultured in a humid atmosphere containing 5% CO_2 at 37 °C for 6 and 24 h. Controls include KSL-W untreated epithelial cell cultures, and epithelial cells treated with heat-inactivated (at 120 °C for 30 min) KSL-W. Following each time point, the culture supernatants were removed and the cultures were washed twice with warm medium, with adherent cells subjected to Hoechst staining assay. The cells were incubated with 2 μg/ml of Hoechst 33342 (H42) in PBS at room temperature for 15 min and were then washed extensively with PBS after which time the stained cells were observed and photographed under an epifluorescence microscope.

2.4. Effect of KSL-W on gingival epithelial cell viability/growth

Gingival epithelial cells (4×10^4 cells/well) were cultured in appropriate medium in the presence of various concentrations (12.5, 50 and 100 μg/ml) of KSL-W. Controls include KSL-W untreated epithelial cell cultures, and epithelial cells treated with heat-inactivated (at 120 °C for 30 min) KSL-W. Monolayer cultures were maintained in a 5% CO_2 humid atmosphere at 37 °C for 24 and 72 h. Thereafter, cell proliferation was assessed using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay (Sigma, St. Louis, MO, USA), which measures cell growth as a function of mitochondrial activity [20]. MTT assay is based on tetrazolium ring hydrolysis by mitochondrial dehydrogenase, which results in formazan, an insoluble blue reaction product. Briefly, a stock solution (5 mg/ml) of MTT was prepared in PBS and was added to each culture at a final concentration of 1% (v/v). Gingival epithelial cell cultures were incubated at 37 °C for 4 h with the MTT solution. At the end of this culture period, the supernatant was removed and the adherent cells were washed twice using warm culture medium. Following the last wash, 2 ml of 0.04 N HCl in isopropanol was added to each culture well, and the incubation was extended for another 15 min. After this step, 200 μl (in triplicate) of the reaction mixture was transferred to a 96-well flat-bottom plate and the absorbance (optical density, OD) was measured at 550 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Model 680, Bio-Rad Laboratories, Mississauga, ON, Canada). Results are reported as the means ± SD of six separate experiments.

2.5. Growth of KSL-W-treated C. albicans on EHOM

C. albicans (SC5314) was inoculated into 10 ml Sabouraud dextrose broth (Difco; Becton-Dickinson) supplemented with 0.1% glucose and was grown to the stationary phase overnight at 30 °C in a shaking water bath. Following culture, blastoconidia were collected, washed with PBS, counted by means of a hemocytometer [38], and adjusted to 10^7 cells/ml. *C. albicans* (10^7 cells in 1 ml of Sabouraud medium) was then incubated for 24 h with KSL-W at 0, 12.5, 50, and 100 μg/ml, at 37 °C. As a positive control, *C. albicans* was also cultured in the presence of amphotericin B at 5 μg/ml. For the negative control, *C. albicans* was cultured in the presence of 100 μg/ml of heat-inactivated (120 °C for 30 min) KSL-W peptide. Following culture with or without KSL-W, *Candida* cells were washed twice with PBS then 10^6 cells in 200 μl of Sabouraud dextrose broth were overlaid on the top of the EHOM. After culture for 24 h, the EHOMs were flipped onto Sabouraud dextrose agar

Table 1
Primer sequences used for the q-RT-PCR.

Gene name	Primers sequences	Product size (bp)
TLR-2	Sense: 5′-GCCTCTCCAAGGAAGAATCC-3′ Antisense: 5′-TCCTGTTGTTGGACAGGTCA-3′	144
TLR-4	Sense: 5′-AATCTAGAGCACTTGGACCTTTCC-3′Antisense: 5′-GGGTTCAGGGACAGGTCTAAAGA-3′	116
TLR-6	Sense: 5′-CATCCTATTGTGAGTTTCAGGCAT-3′ Antisense: 5′-GCTTCATAGCACTACATCCCAAG-3′	121
TLR-9	Sense: 5′-GGACCTCTGGTACTGCTTCCA-3′ Antisense: 5′-AAGCTCGTTGTACACCCAGTCT-3′	151
β1-Defensin	Sense: 5′-GCCTCTCCCCAGTTCCTGAA-3′ Antisense: 5′-GCAGAGAGTAAACAGCAGAAGGTA-3′	82
β2-Defensin	Sense: 5′-TGTGGTCTCCCTGGAACAAAAT-3′ Antisense: 5′-GTCGCACGTCTCTGATGAGG-3′	105
β3-Defensin	Sense: 5′-CTTCTGTTTGCTTTGCTCTTCCT-3′ Antisense: 5′-CTGTTCCTCCTTTGGAAGGCA-3′	138
β4-Defensin	Sense: 5′-CACTCTACCAACACGCACCTAG-3′ Antisense: 5′-CGCAACTGGAACCACACACT-3′	133
GAPDH	Sense: 5′-GGTATCGTCGAAGGACTCATGAC-3′Antisense: 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′	180

plates and left in place for 5 min. The EHOMs were then removed and the plates were incubated for 24 h at 30 °C, after which time the colony-forming-units (CFU) were counted. Results are reported as the means ± SD of six separate experiments.

2.6. Effect of KSL-W-treated C. albicans on TLR and β-defensin gene expression by gingival epithelial cells in the EHOM

C. albicans (SC5314) was incubated in the presence or absence of active KSL-W at various concentrations (12.5, 50 and 100 μg/ml) or heat-inactivated KSL-W (100 μg/ml) for 24 h at 37 °C prior to use to infect the EHMO, as described above. *C. albicans* (10^6 cells in 200 μl of Sabouraud medium) was laid on top of each engineered mucosa sample. The tissues were then incubated in a 5% CO_2 humid atmosphere for 12 h at 37 °C. Following incubation, each EHOM tissue was washed twice with fresh medium and thereafter, the epithelia were detached from the lamina propria and used to extract total RNA.

2.7. RNA extraction and quantification

Total cellular RNA was extracted using the Illustra RNAspin Mini (GE Health Care UK Limited, Buckingham, UK). The concentration, purity, and quality of the isolated RNA were all determined using the Experian system and RNA StdSens analysis kit according to instructions provided by the manufacturer (Bio-Rad, Hercules, CA, USA).

2.8. Quantitative real-time RT-PCR

RNA (1 μg of each sample) was reverse transcripted into cDNA using Maloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Life Technologies, Mississauga, ON, Canada) and random hexamers (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, QC, Canada). The conditions for the preparation of the cDNA templates for PCR analysis were 10 min at 65 °C, 1 h at 37 °C, and 10 min at 65 °C. Quantitative PCR (qPCR) was carried out as previously described [3]. Amounts of mRNA transcripts were measured using the Bio-Rad CFX96 real-time PCR detection system. Reactions were performed using a PCR supermix from Bio-Rad (iQ SYBR Green supermix). Primers (Table 1) were added to the reaction mix at a final concentration of 250 nM. Five microlitres of each cDNA sample was added to a 20 μl PCR mixture containing 12.5 μl of iQ SYBR Green supermix (Bio-Rad) and 0.5 μl of specific primers (TLR2, TLR4, TLR6, TLR9, HBD1, HBD2, HBD3, HBD4, or GAPDH) (Medicorp, Inc., Montréal, QC, Canada) and 7 μl of RNase/DNase-free water (MP Biomedicals, Solon, OH, USA). Each reaction was performed in a Bio-Rad MyCycler Thermal Cycler. For the qPCR, the CT was automatically determined using the accompanying Bio-Rad CFX manager. The thermocycling conditions for the TLRs were established as 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at72 °C, with each reaction done in triplicate. For the HBDs, the thermocycling conditions were 3 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 10 s at 63 °C, and 30 s at 72 °C, with each reaction performed in triplicate. The specificity of each primer pair was verified by the presence of a single melting temperature peak. GAPDH produced uniform expression levels varying by less than 0.5 CTs between sample conditions and was therefore used as a reference gene for this study.

2.9. Western blot analyzes of TLR production following EHOM contact with KSL-W-treated C. albicans

Following contact with KSL-W treated *Candida* for 24 h, as described above, each EHOM tissue was washed twice with fresh medium and thereafter, the epithelia were detached from the lamina propria and total proteins were extracted using lysis buffer (50 mM of HEPES, pH 7.4; 1% (v/v) Triton X-100; 4 mM of EDTA; 1 mM of sodium fluoride; 0.1 mM of sodium orthovanadate; 1 mM of tetrasodium pyrophosphate; 2 mM of phenylmethylsulfonyl fluoride; 10 μg/ml of leupeptin; and 10 μg/ml of aprotinin). The lysates were incubated for 60 min on ice and then vortexed, with the insoluble materials removed by centrifugation (14,000 × *g*, 2 min, 4 °C). The protein lysates were subsequently used for immunoblotting. Whole cell lysates (40 μg of total protein) were subjected to SDS polyacrylamide gel electrophoresis and were electroblotted onto PVDF membranes (pore size 0.2 μm). The membranes were blocked with 5% BSA in Tween-20/Tris-buffered saline (TBS) (TTBS; Tris-buffered saline, 0.1% Tween-20) for 1 h at ambient temperature and were incubated overnight at 4 °C with primary anti-TLR2 (1:250, Imgenex, San Diego, USA), anti-TLR4 (1:200, Imgenex, San Diego, USA), anti-TLR6 (1:200, R&D Systems, Minneapolis, USA), anti-TLR9 (1:500, Cell Signaling, Danvers, MA, USA) and anti-βactin (1:5000) antibodies in TTBS containing 0.5% BSA. The membranes were washed three times with TTBS for 10 min after which time they were incubated with horseradish peroxidase labeled secondary antibody (1:1000 in TTBS) for 1 h at ambient temperature. For protein detection, the membranes were washed for 3 h with TBS, were subsequently incubated in ECL (Amersham), and were assessed by means of a FujiFilm Image Reader LAS-1000 Pro (FujiFilm USA, Valhalla, NY, USA). The experiment was repeated four separate times.

2.10. Measurement of IL-1β, IL-6, and IL-8 secretion following interaction of KSL-W-treated C. albicans with the EHOM

To determine the protein levels of cytokines IL-1β, IL-6, and IL-8, supernatants obtained from the EHOM following their infection with KSL-W-treated *C. albicans* for 24 h were analyzed by sandwich enzyme-linked immunosorbent assays (ELISA, R&D System, Minneapolis, MN). The supernatants were collected in tubes containing 1 μl of a protease inhibitor cocktail for specific use with mammalian cell and tissue extracts (Sigma–Aldrich). Immediately after, the supernatants were filtered through 0.22 μm filters and used either undiluted or at a dilution of 1/50 in PBS

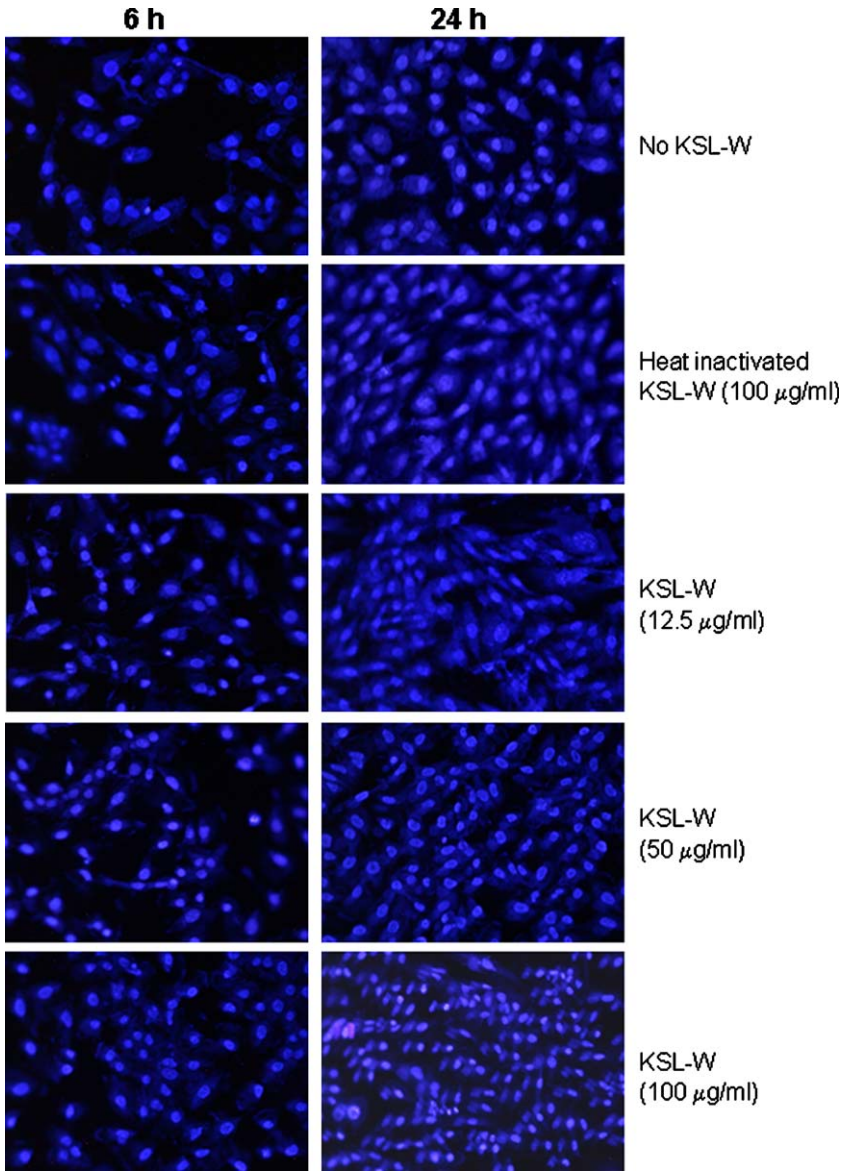

Fig. 1. Photomicrograph of the epithelial cell monolayer cultures following treatment with KSL-W. No effect on cellular adhesion was observed after 6 and 24 h culture periods. Photos are representative of five separate experiments.

to measure cytokine levels. ELISA plates were read at 450 nm and were analyzed using a Microplate Reader Model 680 (Bio-Rad, USA). The minimum detectable concentrations were under 1 pg/ml for the IL-1β, 0.7 pg/ml for the IL-6, and 3.5 pg/ml for the IL-8, as reported by the manufacturer. Each experiment was repeated four times and the means ± SD were calculated and presented.

2.11. Statistical analyzes

Each experiment was performed at least four times, with experimental values expressed as means ± SD. The statistical significance of the differences between the control (absence of KSL-W) and the test (presence of KSL-W) values was determined by means of a one-way ANOVA. *Posteriori* comparisons were done using Tukey's method. Normality and variance assumptions were verified using the Shapiro-Wilk test and the Brown and Forsythe test, respectively. All of the assumptions were fulfilled. *P* values were declared significant at ≤ 0.05. Data were analyzed using the SAS version 8.2 statistical package (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. KSL-W had no inhibitory effect on gingival epithelial cell adhesion

Fig. 1 shows that KSL-W had no effect on cell morphology at concentrations ranging from 12.5 to 100 μg/ml as well as no effect on the adhesion of cultured epithelial cells. In addition, no difference in cell density was observed in the epithelial cells cultured in the presence or absence of KSL-W. Heat-inactivated KSL-W has no effect on epithelial cell adhesion (Fig. 1). These results clearly demonstrate the absence of any toxic effect of KSL-W on the adhesion of cultured epithelial cells.

3.2. KSL-W had no side effect on gingival epithelial cell proliferation

As shown in Fig. 2, epithelial cell growth at 24 h post-treatment with KSL-W was comparable to that recorded by the untreated culture, with an optical density ranging from 0.7 to 0.8. After 72 h

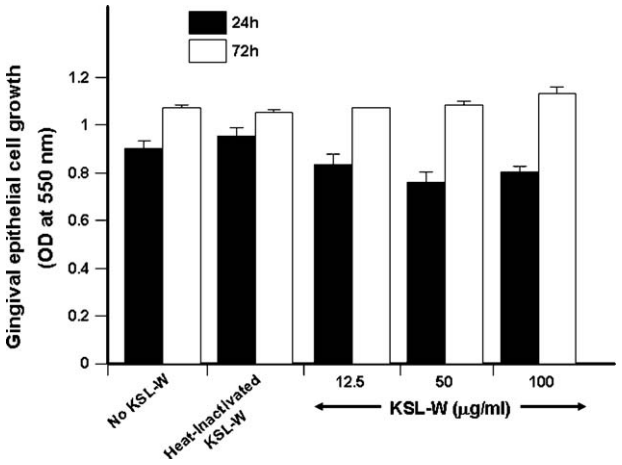

Fig. 2. Effect of KSL-W on human gingival epithelial cell proliferation. Following epithelial cell culture in the presence or not of KSL-W for 24 and 72 h, each culture was subjected to an MTT assay. Cell proliferation was assessed by means of absorbance measurements. Note that no effect on cellular proliferation was observed in the presence of different test concentrations of KSL-W antimicrobial peptide. Heat-inactivated KSL-W has also no effect on cell growth.

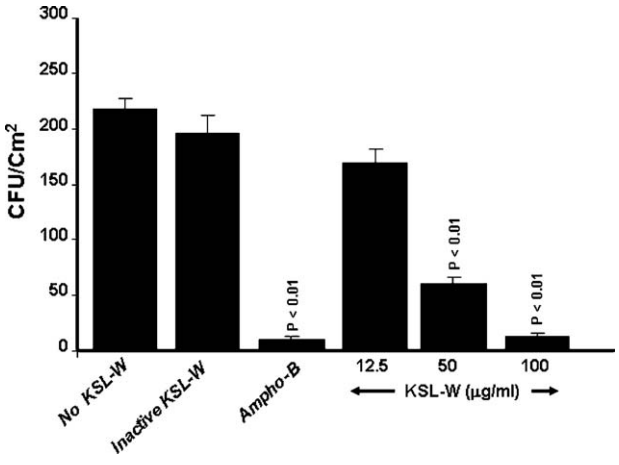

Fig. 3. Adhesion and growth of KSL-W-treated *C. albicans* on the EHOM. The pre-treatment of *C. albicans* decreased its adhesion and growth on the EHOM. KSL-W-untreated *Candida*, *Candida* treated with 5 µg/ml of amphotericin B are shown for comparison with 12.5, 50 and 100 µg/ml KSL-W pre-treated *Candida*. Results represent means ± SD of four different experiments.

of culture, epithelial cell growth increased equally in the control group and the KSL-W-treated group. Heat-inactivated KSL-W did not affect epithelial cell proliferation (Fig. 2).

3.3. KSL-W pre-treatment reduced Candida growth in the infected oral mucosa tissue

Fig. 3 shows that the growth of *C. albicans* was significantly inhibited by the KSL-W pre-treatment, compared to that of the untreated control. A dose response of the KSL-W pre-treatment was also observed, as the number of CFUs recovered decreased from 170 ± 13 CFUs/cm^2 to 12 ± 2 CFUs/cm^2 when the *Candida* cells were pre-treated with 12.5 µg/ml and 100 µg/ml of KSL-W, respectively. Heat-inactivated KSL-W showed no effect on *Candida* growth (Fig. 3).

3.4. KSL-W modulated TLRs expression by the EHOM gingival epithelial cells following contact with C. albicans

RT-PCR results indicate that when the EHOM was cultured in the presence of KSL-W-treated *C. albicans*, TLR2 mRNA expression was

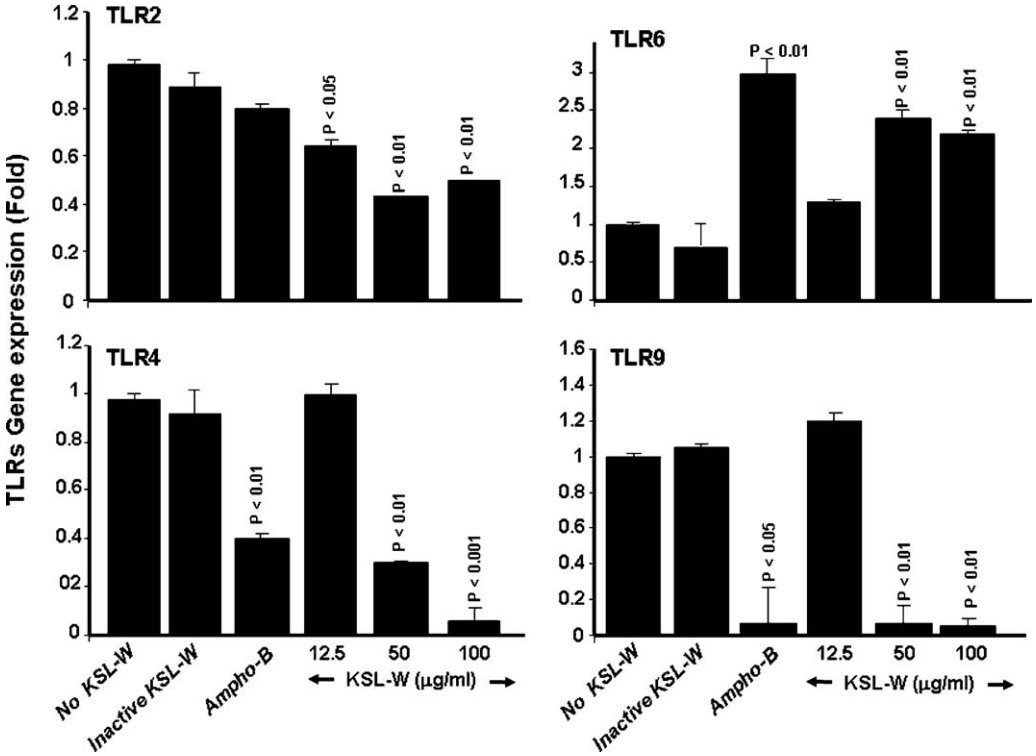

Fig. 4. TLR expression by gingival epithelial cells following infection by KSL-W-treated *C. albicans*. Pre-treating *C. albicans* with KSL-W reduced its ability to induce TLR2, 4, 6, and 9 gene expression. Results are presented as the number of fold increase in expression of the genes in the test samples compared to that in the control. Data are expressed as means ± SD from triplicate assays of three different experiments.

reduced by approximately five fold as compared to the expression level exhibited by the EHOM cultured in the presence of untreated *Candida*. This reduction occurred in a dose-dependent manner (Fig. 4). Similar results were obtained with TLR4 expression. When *C. albicans* was pre-treated with KSL-W, TLR4 mRNA expression by the epithelial cells significantly decreased, contrasting that of the EHOM epithelial cells infected with untreated *Candida*. This decrease in expression level appeared to be dose-dependent (Fig. 4). Thus KSL-W reduced *C. albicans* virulence, leading to a down regulation of TLR4 expression by the EHOM epithelial cells. TLR9 is also a key receptor implicated in pathogen recognition by epithelial cells. *C. albicans*-treated KSL-W significantly reduced TLR9 mRNA expression by the EHOM compared to the expression recorded by the tissues infected with untreated *C. albicans* (Fig. 4). This significantly inhibited expression was observed by the pre-treatment of *Candida* with KSL-W at 50 and 100 μg/ml. In contrast to the previously described TLRs, TLR6 expression was up regulated when the EHOM was infected with KSL-W-treated *C. albicans* (Fig. 4). The increased TLR6 mRNA expression was obtained with 50 and 100 μg/ml of KSL-W and was comparable to the expression obtained with the ampho-B-treated *C. albicans*, suggesting that both KSL-W and ampho-B modulated TRL6 mRNA expression by the gingival epithelial cells in a similar fashion. As shown in Fig. 4, KSL-W and ampho-B had the same effect on TLR2, TLR4, and TLR9 mRNA expression by the EHOM gingival epithelial cells following *C. albicans* infection. Heat-inactivated KSL-W pre-treated *Candida* showed no effect on the expression of TLRs by EHOM epithelial cells (Fig. 4). Protein analyzes using Western blot (Fig. 5) confirmed the mRNA expression results. Indeed, TLR2, TLR4 and TLR9 proteins were reduced in EHOMs infected with KSL-W treated *Candida* (Fig. 5). In contrast, TLR6 protein was increased basically with 50 and 100 μg/ml of KSL-W (Fig. 5).

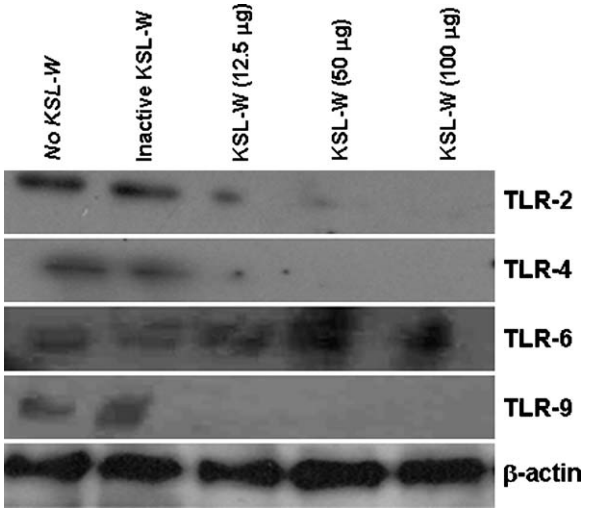

Fig. 5. TLR protein production by gingival epithelial cells following infection by KSL-W-treated *C. albicans*. Western blot analysis of TLR2, TLR4, TLR6 and TLR9 proteins following EHOMs contact with untreated and KSL-W treated *Candida* ($n = 4$).

3.5. KSL-W downregulated the effect of C. albicans on human β-defensin expression

As shown in Fig. 6, when *Candida* was pre-treated with KSL-W at 12.5 and 50 μg/ml, HBD1 mRNA expression was unchanged, yet it was reduced in the EHOM gingival epithelial cells when treated with KSL-W at 100 g/ml. Similarly, *Candida* pre-treated with KSL-W at 50 and 100 μg/ml reduced HBD2 mRNA expression by the epithelial cells (Fig. 6). Also, EHOM infected with KSL-W pre-treated

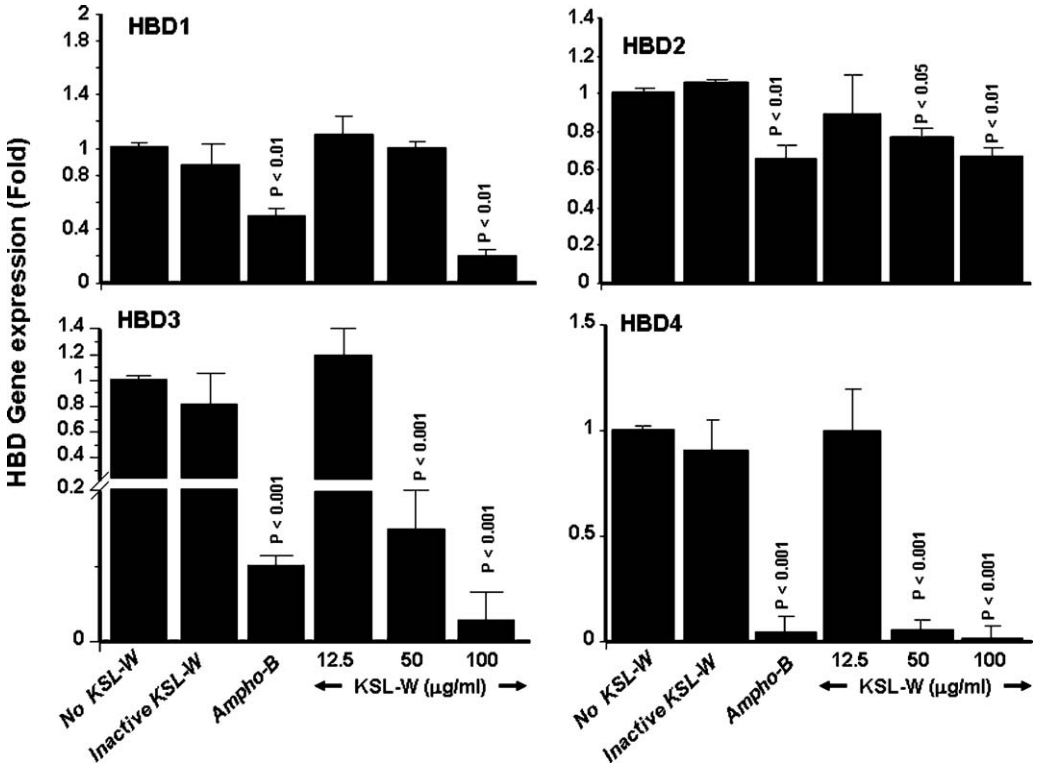

Fig. 6. β-Defensin expression gingival epithelial cells following contact with KSL-W pre-treated *C. albicans*. Pre-treating the yeast with KSL-W resulted in reduced HBD1, HBD2, HBD3, and HBD4 gene expression by the EHOM epithelial cells following infection. The decreased level of β-defensin expression is similar to that observed with the amphotericin B-treated yeast cells. Results are presented as the number of fold increase in expression of the genes in the test sample compared to the gene expression in the control. Data are expressed as means ± SD from triplicate assays of three different experiments.

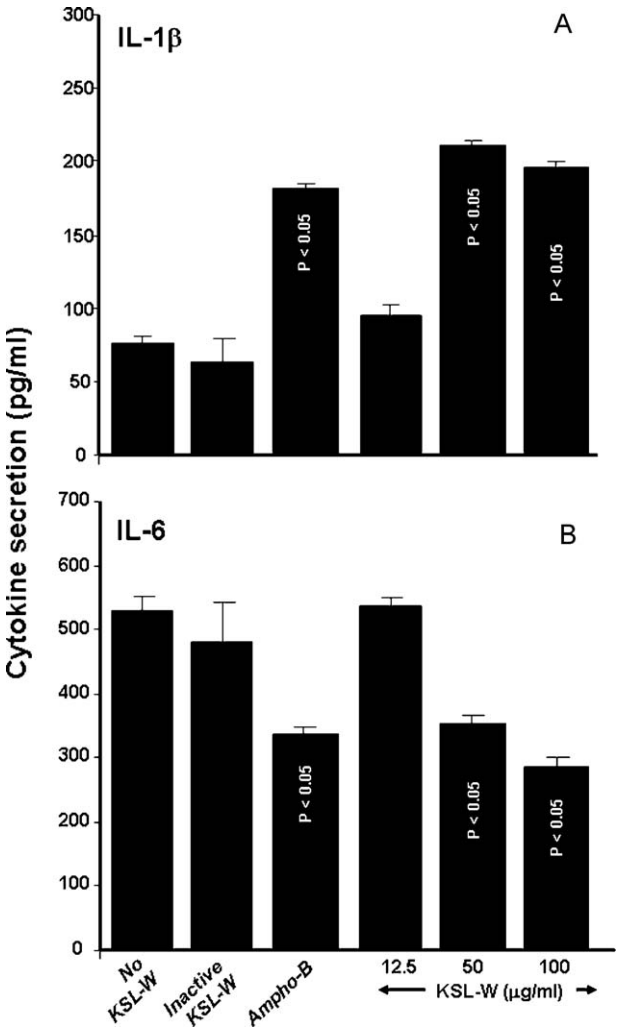

Fig. 7. Cytokine secretion by the gingival epithelial cells following *C. albicans* infection. Increased IL-1β (A) and decreased IL-6 (B) secretions were observed in the EHOM gingival epithelial cells infected with KSL-W pre-treated *C. albicans*. KSL-W at high concentrations, and amphotericin B effects were comparable. Data are the means ± SD of four separate experiments.

C. albicans, showed reduced levels of both HBD3 and HBD4 mRNA expression (Fig. 6). It should be noted that the antifungal molecule ampho-B had an effect comparable to that of KSL-W on HBD1, 2, 3, and 4 mRNA expressions (Fig. 6). Heat-inactivated KSL-W pre-treated *Candida* showed no effect in human β-defensins expression.

3.6. Cytokine induction

As shown in Fig. 7A, following EHOM infection with KSL-W pre-treated *C. albicans* for 24 h, the secretion of IL-1β was significantly increased basically with 50 and 100 μg/ml of KSL-W. This effect was similar to the one obtained with ampho-B (Fig. 7a). Inactivated KSL-W pre-treated *Candida* showed similar effect as non-infected EHOM. By contrast, the secretion of IL-6 significantly decreased after 24 h of contact of EHOM with the KSL-W pre-treated *Candida* (Fig. 7B). Decreases of IL-6 secretion was important when KSL-W was used at 50 and 100 μg/ml. At high concentration, the KSL-W effect was comparable to the one obtained with ampho-B-treated *Candida* treated EHOM. A study performed on IL-8 secretion showed no significant modulation of this chemokine. Results suggest that KSL-W may potentially modulate cell defenses against infection through IL-1β and IL-6. When a higher concentration (100 μg/ml) of KSL-W was used, the effect was also comparable to that of ampho-B. However, the effects of KSL-W on IL-1β and IL-6 secretions are different.

4. Discussion

Antimicrobial peptides displaying *in vitro* activity against human bacterial pathogens [28] have been proposed as a novel control strategy to combat bacterial infections [27,34] and an alternative solution to control fungal infections [27,28,34]. As some available antifungal agents are ineffective (in terms of resistance) and may cause significant side effects, the medical community is now turning its interest toward new anti-fungal molecules. Naturally occurring antimicrobial peptides (AMPs) have been shown to play an essential protective role against microbial infections in species across the animal and plant kingdoms.

Synthetic peptides that mimick many of the key structural features essential for the antimicrobial activity of naturally occurring AMPs have emerged as substitutes against microbial infections. Our laboratories have focused on the antimicrobial activity of synthetic decapeptide KSL and its structural analog KSL-W. We in fact discovered that these peptides possess potent antimicrobial activity against a wide variety of oral and non-oral bacterial pathogens [6,10]. However, their potential effect against fungi, such as *C. albicans*, remains to be determined.

Oral candidiasis is associated with gingival tissue where epithelial cells are the primary components of the oral mucosa [11,16,38]. We sought to determine the effect of antimicrobial peptide KSL-W in controlling oral candidiasis and its toxicity, if any, against oral mucosa cells. Our results show that at both low and high concentrations, KSL-W had no effect on the adhesion and proliferation of monolayer cultures of gingival epithelial cells. These findings, reported here for the first time, demonstrate that decapeptide KSL-W exhibited no toxicity against human gingival epithelial cells.

To determine the potential effect of antimicrobial KSL-W against oral candidiasis, we produced an engineered oral mucosa to mimick the in vivo conditions in which the yeast is in contact with the host tissue. In this context, engineered oral mucosa was cultured in the presence of *C. albicans* which was pre-treated or not with various concentrations of antimicrobial peptide KSL-W. We found that KSL-W downregulated the adhesion and growth of *C. albicans* on the EHOM. Compared to the control, the number of *C. albicans* CFUs had significantly decreased following treatment with KSL-W at concentrations of 50 and 100 μg/ml. These results therefore demonstrate the growth-inhibiting effect of KSL-W against *Candida*. Similar results were previously observed using other antimicrobial peptides such as human β-defensins [8,14]. Although we did demonstrate that antimicrobial peptide KSL-W may inhibit *Candida* growth, the mechanism by which it reduces this growth remains to be determined.

It is well documented that epithelial cells play an active role in protecting the host against invading microbial pathogens. Recent studies have demonstrated the possible involvement of TLRs in the recognition, by epithelial cells, of fungal pathogens including *Candida* species [2,19,26]. The present study links TLR activation in oral epithelial cells to *C. albicans* infection using a human oral mucosa model and the ability of KSL-W to attenuate the capacity of *Candida* activating the innate immunity.

Quantitative real-time RT-PCR revealed that the gingival epithelial cells expressed TLR2, TLR4, and TLR9 following infection with *C. albicans*. However, following KSL-W treatment of the yeast, the expression of these TLRs from the targeted epithelial cells was downregulated. These data support those showing that epithelial cells respond to microbial infections through TLRs [2,22,42]. As in the case of antifungal drugs such as ampho-B, KSL-W down-

regulated *Candida* virulence, which in turn modulated the innate response of the epithelial cells through decreased TLR2, TLR4, and TLR9 gene and protein expression.

It remains unclear whether the decreased TRL expression by the epithelial cells was due to the change of surface phenotypes or the loss of viability of *Candida* following pre-treatment with KSL-W. These changes may in fact contribute to its reduced ability to elicit host innate responses. On the other hand, the observed results may also indicate the ability of epithelial cells to sense and distinguish differences between microbial challenges encountered (for example, virulent versus avirulent strains or dead organisms), resulting in various levels of subsequent activation of host innate responses.

Further studies will elucidate the underlying mechanistic interaction(s) between KSL-W treated *Candida* and gingival epithelial cells that lead to this decrease in TLR expression. Of interest is that while a decrease was observed in TLR2, TLR4, and TLR9 expression due to pre-treatment with the peptide, TLR6 expression increased in the targeted cells. Similar results were obtained by treating specific organisms with natural molecules, such as garlic [44], and quorum-sensing inhibitory molecules, such as farnesol [8].

Recognition of pathogen-associated molecular patterns of invading microbial organisms by epithelial cells through TLRs can lead to the expression, by these cells, of native antimicrobial peptides such as HBDs [43]. In this study, we confirm that gingival epithelial cells may prevent *C. albicans* infection by expressing various HBDs. Interestingly, the KSL-W-treated *C. albicans* demonstrated less virulence as evidenced by a reduced activation of epithelial cells through reduced HBD expression. This effect was similar to that obtained with antifungal agents, such as amphotericin B, which are used clinically to control *Candida* infection. These results suggest that KSL-W attenuated *Candida* virulence, thus when it came in contact with epithelial cells, the latter were able to sense the avirulent nature of the challenge, which led to a reduced expression of HBDs as a part of the host's inflammatory response to infection.

In addition to the role epithelial cells have in controlling *Candida* pathogenesis through the expression of native antimicrobial peptides, several studies have reported that these cells display innate immunity against bacterial and yeast infections via an active inflammatory process that involves a variety of mediators including IL-1β, IL-6, and IL-8 [12,38,39]. Given the biological role of oral epithelial cells as active immunocytes [4] because of their ability to secrete several pro-inflammatory cytokines, our study shows that the production of both IL-1β and IL-6 were modulated following contact with the KSL-W-treated *C. albicans*. The peptide increased IL-1β, but decreased IL-6 secretions. These effects of KSL-W were comparable to that obtained with amphotericin B. Our data suggest that KSL-W modulated the host pro-inflammatory responses against *C. albicans* through different ways involving IL-1β and IL-6. Further investigations will therefore be necessary to identify the signaling pathway activated by KSL-W-treated *Candida* which results in a selective increase/decrease of IL-1β and IL-6 production.

5. Conclusion

Our study confirms the non-toxic effect of KSL-W on normal human gingival epithelial cells and its ability to inhibit the adhesion and growth of *C. albicans* on engineered human oral mucosa. KSL-W was also shown to reduce *C. albicans* virulence/pathogenesis by reducing *C. albicans* ability to activate the innate immunity exhibited by the targeted epithelial cells. Overall results suggest that KSL-W may have antipathogenic properties and may show potential as an antifungal molecule to treat infections such as oral candidiasis.

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